

PHOTOPERIOD CONTROL OF DIAPAUSE IN *DAPHNIA*. IV.
LIGHT AND CO₂-SENSITIVE PHASES WITHIN
THE CYCLE OF ACTIVATION

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The continual involvement of photoperiod in the life cycle of arthropods is demonstrated by the influence of photoperiod on both the initiation and termination of diapause (Adkisson, 1965). Photoperiod control of diapause in insects has been demonstrated for larval (Paris and Jenner, 1959; Wellso and Adkisson, 1964), pupal (Williams and Adkisson, 1964) and imaginal stages (deWilde, Duintjer and Mook, 1959). Embryonic diapause is also known to be initiated in response to photoperiodic induction. This has been demonstrated for a number of insects and for the Crustacean, *Daphnia pulex* (Stross and Hill, 1968).

In previous study with *Daphnia* the termination of diapause may require a light stimulus (Stross, 1966, 1969), thereby demonstrating the presence of a photoreceptor which may be absent in early embryos of some insects (Minis and Pittendrigh, 1968). Furthermore, the light stimulus may need to be "long-day," although many embryos are activated in "short-days" as well. Since photoperiod control of diapause initiation may be controlled by density of the experimental animals, *i.e.*, facultative, the suggestion is that an unknown stimulus present in the water may interfere with the retention of diapause in short daylengths. The present study was undertaken to demonstrate photoperiod control of diapause termination in *Daphnia*.

A second objective of the study was to test the applicability of two empirical models of photoperiodism, a test made possible by the remarkable feature of the diapaused embryo of *Daphnia* to respond to a single light period. Bunning (1959, 1964) described a physiological rhythm of maximum light sensitivity, the timing of which he suggested may be more influenced by the "dawn" than by the "sunset" of a natural or simulated day. A restatement of the model was made possible by a more explicit knowledge of the action of light on a circadian rhythm in an insect. The restated (coincidence) model (Pittendrigh and Minis, 1964) argues that the so-called time or phase of maximum sensitivity to light (photo-inducible phase) is coupled to the circadian cycle of the organism. Thus the light period of a daily cycle phases the circadian cycle, and when the light period is "long-day," it illuminates the organism at a time when it is photo-inducible. The dual effect of light is operationally significant when the experimental organism requires more than one inductive cycle. The coincidence model is satisfied when the long-day response is induced by a single pulse of light per cycle administered to an organism at the photo-inducible phase of its circadian cycle. The position of the inducible phase was first indicated to be near sunset (Pittendrigh and Minis, 1964)

and later generalized to include the possibility that it could be at dawn (Pittendrigh, 1966).

An alternative model of photoperiodism (Lees, 1966) suggests that time measurement is restricted to the dark period, and a long-day condition results (virginoparae) when the night is critically short (Lees, 1966). In an organism requiring but a single period of light for induction, two pulses of light suitably spaced with respect to one another but independent of the organism's circadian cycle would suffice to induce the long-day condition. This so-called "interval-timer" has been tested with consistent results in the aphid *Megoura*. A recent study (Hamner, 1969) with a moth (*Carpocapsa*) strongly indicates the presence of both a circadian and an interval-timer in the photoperiodic response.

Previous study has shown that the diapaused embryo of *Daphnia* contains a minimum of two phases, a photo-refractory phase followed by a photo-sensitive phase (Stross, 1965). The photo-refractory phase in embryos collected from the wild is completed with exposure to low temperature; 4° C was found adequate for embryos collected at temperate latitudes (Stross, 1966, 1969) but a lower temperature was necessary for embryos of *D. middendorffiana* collected from the arctic (unpublished). The duration of the photo-refractory phase may be different in summer and winter diapausing strains, and much shorter in the former (Stross, 1969). The alternative condition of having the same or similar thermal optimum for both the active and diapause states has also been shown to exist in Supply House cultures of the species (Stross, 1966; Davison, 1969).

The photo-sensitive phase in *Daphnia pulex* may or may not require light for terminating the embryonic diapause. In a winter diapausing strain, a light requirement is restricted to two situations: pre-mature termination in autumn before the diapause state intensifies, and in the spring when the embryos are incubated in a crowded and presumably oxygen deficient environment (Stross and Hill, 1968). In other strains including the ones lacking a low temperature optimum, light remains an absolute requirement, that is the diapause is maintained indefinitely in constant darkness. The suggestion that the process underlying activation may be basically photoperiodic has been apparent in both light-escaping and light-requiring strains (Stross and Hill, 1968; Stross, 1969).

METHODS AND MATERIALS

The egg pods (ephippia) containing usually a pair of diapaused embryos were removed from the bottom of the culture vessel and transferred to constant dark. They were mass incubated while either lying at the bottom of beakers (stagnant environment) covered with saran or suspended in nylon net within a flowing stream (Fig. 1). The embryos were mass produced in cultures of a strain of *Daphnia pulex* Leydig that came originally (1960) from a Biological Supply House. The strain is capable of completing the photo-refractory phase at 20° C (Stross, 1966), the incubation temperature in these experiments, although the environment may require other modification (see Results). The strain could be classed as the dicyclic type since at room temperature (21°) the females readily reproduce exclusively the diapausing embryos when densely cultured in long day-lengths. The cultures were maintained in room light supplemented with fluorescent lighting on an L18:D6 regimen.

At the time of light exposure embryos were transferred under safelight to individual vials (25 × 95 mm) containing 20 ml of medium which was either lake water or a synthetic substitute (Stross and Hill, 1968). Ten or 25 egg pods containing a determined number of embryos were placed into each vial. The vials were then covered with saran or, as in the gasing experiments, stoppered with a rubber stopper lined with saran. Vials were prepared by autoclaving in a strong bicarbonate solution followed by rinsing in distilled water; this treatment was found to reduce the variance which can be a serious problem in hatching experiments. The embryos were exposed to "cool-white" fluorescent light and at intensities ranging from 1000 to 2000 lux, depending on the experiment, for intervals as indicated.

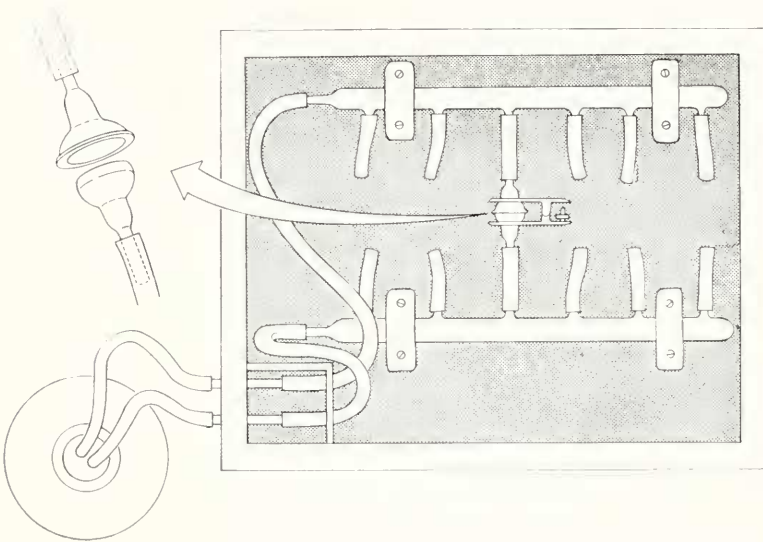


FIGURE 1. A "flowing stream" for incubating diapaused embryos of *Daphnia* in constant darkness. The embryos are held in small cylinders of nylon screen which is inserted into the ball joints between the manifolds.

In the experiments with modified atmosphere, the gas was introduced from prepared compressed sources (Linde-Union Carbide) with a sintered glass diffuser. The diffuser was introduced directly into the vial and the gas bubbled for 1.0 minute. Enriched CO₂ mixtures consisted of 10.0 and 49.0 per cent CO₂, a 20 per cent O₂ concentration, and the balance N₂. A 5.0 per cent CO₂ atmosphere was also used in which the CO₂ had been added to air (*i.e.*, displacing both O₂ and N₂). Enrichment with CO₂ was carried out at the time of light exposure. Preliminary treatment with N₂ was carried out in the same manner, except that masses of embryos were treated in screw-top jars which were sealed following gasing. This procedure may not have removed all O₂ from the medium, although in the CO₂ enrichment the treatment was sufficient to bring the medium to a new pH equilibrium.

RESULTS

Activation of the diapaused embryo of *Daphnia pulex* Leydig may require light received as one long day or its "skeleton" (Fig. 2). In two experiments, light exposure was necessary at two separate intervals within a single light period. Embryos which had been incubated in constant dark and a natural thermocycle were first exposed to light at 0900 EST either for 2 or 16 hours. Those exposed for only two hours required a second exposure and the timing

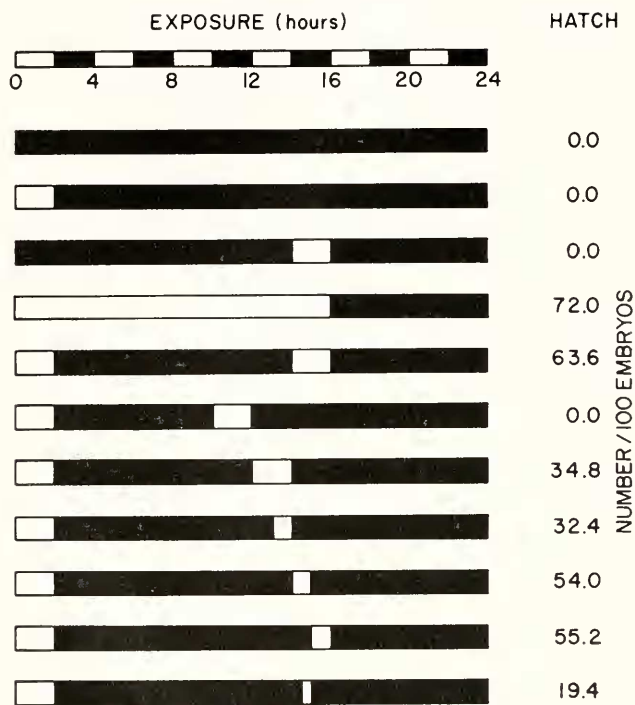


FIGURE 2. Hatching of *Daphnia* embryos when given a single 16-hour light period or two pulses of light that form the skeleton of one 16-hour light period. The embryos were first exposed at 0900 EST after having been in constant darkness and near an open (September) window for 17 or 31 days. Prior to that time the embryos were in constant darkness and temperature (19° C) for four months. (See text for additional details.)

of the second exposure was critical. When exposed to a second pulse from hours 14 to 16 following the start of the first pulse, activation (63.6 per cent), as measured by hatching, was equivalent to a continuous 16-hour light exposure (72.0 per cent). A second exposure from hours 10 to 12 was not effective (0.0 per cent hatch) as was a single exposure given from hours 14 to 16.

Exposure to one 14-hour skeleton resulted in the hatching of $\frac{1}{2}$ (34.8) the number activated by one 16-hour skeleton. This result suggested that the critical photoperiod for the embryos was approximately 14 hours of light. Further deductive support for 14 hours as the critical daylength was given by the number

of embryos activated with a light pulse of shorter duration than the standard two hours. A one-hour pulse from hours 13 to 14 activated as many embryos as the standard pulse given from hours 12 to 14, whereas a one-hour pulse given after hour 14 resulted in the activation of a larger number of embryos. Further reduction in the duration of the light pulse, as for example to one-half hour, reduced activation (Fig. 2) and verified results of preliminary experiments. It is to be emphasized that these results were obtained from exposure of the embryos to a single photoperiod or its skeleton.

The requirement for two exposures appropriately spaced suggested that two photo-inducible phases exist and exposure to both may be a necessary part of long-day induction in *Daphnia* embryos. Lees (1966) has also shown that two exposures per 24 hours were necessary to simulate long daylength in the aphid, *Megoura*, or more precisely, to keep the night interval critically short. The apparent similarity between the two evoked such questions as whether the day or night interval was being measured and whether the position of at least one of the photo-inducible phases recurred at circadian intervals. Further experimentation proved impossible, however.

The long day or two-pulse requirement proved transitory for embryos held in the dark in a stagnant environment. Subsequent tests with the same batch of embryos, but from different containers, revealed first the loss of the requirement for a second pulse followed by a de-synchronization of photo-sensitivity. In the third experiment, designed to locate the time of photo-sensitivity relative to the thermal dawn of the environment, the first two-hour pulse of light activated as many embryos as the skeleton of a 12 or 16-hour photoperiod. In this new state the embryos were not only responsive to a single pulse but showed a time dependent sensitivity to photo-activation. Three groups of embryos were exposed at the onset of thermal rise (0600) in an artificial thermoperiod, or before (0400) or after (1000) the thermal rise. Activation, as measured by hatching, was largest in the embryos exposed to light at 1000 and 83.0 per cent of the embryos hatched (Fig. 3A). Exposed six hours earlier, only 36.0 per cent hatched ($P = 0.01$). This new condition followed only two weeks after the experiments that clearly showed a need for two pulses, and nine days after the embryos were placed in an artificial thermoperiod.

In an effort to locate the "time" of maximum sensitivity to photo-activation, each of 12 groups were exposed to a different two-hour period within the 24-hour thermocycle. After only four additional thermocycles the success of activation had declined from a maximum of 83.0 per cent to approximately one half that number (Fig. 3A). Two groups of embryos showed maximum sensitivity, one exposed at 0800, a time which corresponded with the thermal rise, and a second exposed eight hours later at 1600. Oddly, the total activation of embryos for the two periods of maximum sensitivity was equal to the number of embryos that had been activated at 1000 in the preceding experiment performed four days earlier.

Several interpretations are possible. The simplest seems to be that the embryos have retained only one phase of photosensitivity and that the rhythm of light sensitivity is responding to the thermocycle. One group of embryos seems to have retained a phase relation to the thermal rise, while a second group began

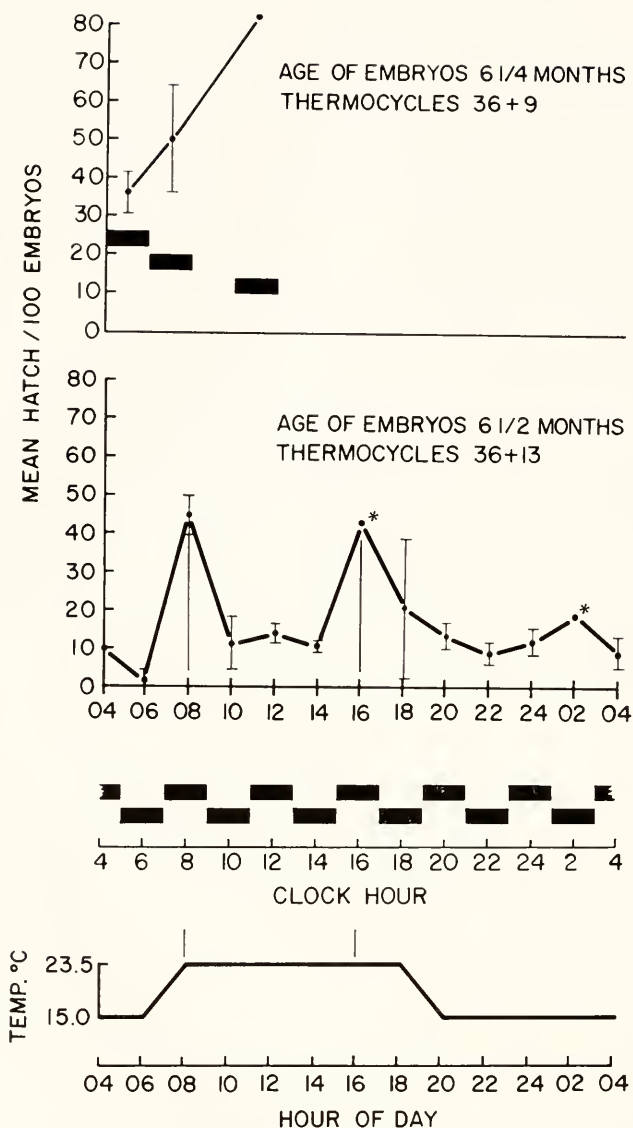


FIGURE 3. Hatching of embryos when given a single two-hour light pulse at various times in an artificial 24-hour thermal cycle. The embryos are from the same batch as shown in Figure 2 but are two weeks or more older. * S.E. is zero.

a synchronous drift to a later time, and at the time of measurement occupied no known relationship to the thermal cycle. The positions of the photo-sensitive phase were also unstable. After an additional month of incubation in the dark, the embryos were activated when exposed at nearly all phases of the thermocycle.

Disclosure of two distinct phases of light sensitivity will form the basis of later interpretation although the condition was unstable. Instability amounted to

the loss of one of two inductive phases requiring light for activation. This was accompanied by the development of an asynchronous and seemingly continual sensitivity to light. The presence of two photo-inductive phases in each thermocycle was also unstable in a second series of experiments with a new batch of diapaused embryos.

In the second series the optimum interval between the two light exposures was clearly a function of when the embryos were first exposed to light pulses of two-hours duration. When exposed at the thermal rise, a 10-hour skeleton photoperiod was more effective than a 16-hour skeleton photoperiod. The reverse was true when the embryos were first exposed 12 hours after the thermal rise. These results are readily interpretable if the embryos become entrained to the thermal oscillations in the environment but do not distinguish the thermal rise (dawn) from the decline (sunset). It would seem that the first pulse of light was exposing the embryos at their "sunset" and the second pulse at "dawn." A delay in the first exposure to light would then result in an initial exposure of the embryos at dawn. In other words the interpretation suggests that the embryos may respond when "seeing" light first at either sunset or at dawn.

Substitution of potential synchronizers other than thermal oscillation was only partially successful. The simple transfer to fresh medium may have been effective. When exposed to a 2-hour pulse at the time of transfer, few (3.3 per cent) embryos were activated. When exposed 24 hours later, 70.0 per cent were activated. Exposure at both times (hours 0 to 2 and 24-26) was ineffective (9.3 per cent). Attempts to synchronize with light were disastrous. Single exposures of 15 minutes to embryos still in the original medium damaged the embryos. A small number (15.6 per cent) hatched but most of the affected (activated?) embryos disintegrated. Hourly exposures to the safelight (red light) at 24-hour intervals were only slightly successful.

The foregoing results demonstrated that light may be required for activation. They clearly show that the *Daphnia* embryo may be discontinuously sensitive to light. Specific times of light sensitivity appear to bear a specific relationship to a natural or imposed thermocycle. Two conditions of photo-sensitivity were revealed. In one condition following the onset of the photo-sensitive phase of diapause, the embryos may require exposure to light at two distinct times within one thermocycle. One of these times may be interpreted as the dawn phase of the embryo's putative daily cycle, the second as the sunset phase. In a second condition the embryo requires only a single exposure to light. Whether the single exposure serves as dawn or sunset cannot be interpreted, as a result of a seemingly ambivalent phase relationship of the embryo's endogenous cycle to the thermocycle of the environment.

Photo-sensitizing with elevated tensions of CO₂

Daphnia embryos held in darkness in a flowing stream (Fig. 1) for up to 14 months retain their viability but are photo-refractory. They may be made photo-sensitive with manipulation of the gaseous environment. Following incubation in a medium bubbled with nitrogen, photo-activation is possible but only when the tension of CO₂ is elevated by bubbling the medium with air enriched with 5.0 per cent CO₂; detailed results are given below. The gas modification is a well

tested procedure for breaking the dormancy of many organisms including certain parasites (Fairbairn, 1961; Rogers and Sommerville, 1968) and seeds (Ballard, 1958, 1967; Ballard and Grant Lipp, 1969). Although some essential details are still lacking, the procedure may guarantee a uniform response from embryos incubated for six months to 14 months in the dark before exposure to light.

The function(s) of CO₂

Elevated CO₂ tensions may perform two functions in the parasite (Sommerville, 1964). One is to break the dormancy and the second is to initiate a development leading to the molt of the diapausing instar. Sommerville (1964, 1966) showed

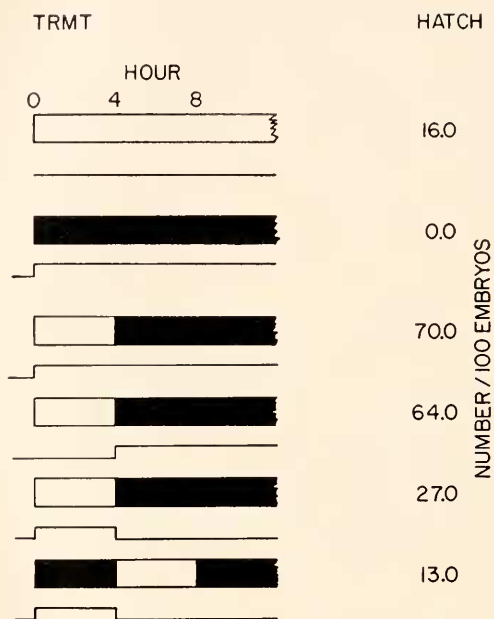


FIGURE 4. Photo-activation in the presence of an elevated CO₂ tension. The medium has been enriched with a mixture of 5.0 per cent CO₂ in air. Embryos were in constant dark and temperature for ten months, six of which were in the "flowing stream" and four months prior to treatment in a medium treated initially with 100 per cent N₂. Note some activation independent of CO₂ enrichment.

the second function could be largely completed within the first 24 hours of incubation. A fungal parasite of cucumbers needs both a light and a dark-requiring process in order to form spores (Barnett and Lilly, 1950, 1955). The dark-requiring process is suppressed by what has been identified as an elevated tension of CO₂ in the culture vessel.

The two functions of CO₂ may not be separable in the activation of *Daphnia* embryos. It is clear that both light and elevated CO₂ tensions participate in the activation process. Moreover, the action(s) of CO₂ is effected in the dark. The exposure of embryos to a four-hour pulse of light either with or before elevation of CO₂ resulted in a high level of activation (Fig. 4). Conversely, pulsing the em-

bryos with CO₂ for four hours either with or before the light pulse had little or no effect. In the experiment the level of hatching in the CO₂ control was significantly greater than zero (16.0 per cent). This background level was eliminated in subsequent experiments by restricting the duration of incubation in nitrogen. Whatever the function of CO₂ in the dark, the presence of CO₂ restricts the light requirement to a maximum of four hours.

A working hypothesis of activation is that the embryos may require stimulation at two separate inductive phases of a daily cycle as shown in earlier experiments (see above). One of the phases has an absolute requirement for light. The second phase might be inducible with light or a substitute such as an elevated tension of CO₂. Results of the first experiment with CO₂ showed that CO₂ renders the embryo photo-sensitive. That CO₂ later substituted for a second pulse of light is an assumption consistent with the dual role of CO₂ as shown by Somerville (1964) and consistent with the observation that CO₂ suppresses a dark (short-day) reaction (Barnett and Lilly, 1955). Thus it may be proposed that CO₂ may have two roles in the activation of the *Daphnia* embryo: to break photo-refractoriness and to substitute for light at one of two photo-inducible phases.

Results supported the hypothesis that CO₂ substitutes for light at one of two inductive phases of a daily cycle, although in a way more striking than anticipated. Ten groups of embryos in triplicate were given an elevated level of CO₂ under safelight. The first group was exposed immediately to a two-hour pulse of light and the other nine were similarly exposed but at a later time in the following 32-hour interval (Fig. 5). Embryos were activated in all but the control groups and ranged from 44 to 100 per cent with no trend apparent. That is the embryos would appear to be photo-inducible at all times tested.

The striking features of hatching were the synchrony and the length of the interval from light exposure to the time of hatching (Fig. 5). The interval assumed two basic patterns. When the embryos were exposed to a light pulse at any of four times in the first 12 hours, they hatched simultaneously and 83 hours after CO₂ elevation, that is they behaved as though CO₂ was the stimulus triggering activation. Controls clearly showed that both light exposure and CO₂ elevation were necessary for activation. In the subsequent 12-hour interval, that is from hours 12 to 24 following CO₂ elevation, the pattern of hatching suggested that light now provided the key stimulus. The situation reversed itself in the third 12-hours and once again the pattern of hatching suggested CO₂ was the key stimulus.

The observed pattern of hatching strongly suggests that CO₂ triggers a dark reaction. It may be supposed that light given during this phase initiates no immediate effect but that the light stimulus is "stored" until the beginning of a second phase at which time a second necessary process is initiated by light following which the embryo begins an irreversible development. If the embryos are not exposed to light until the second 12-hour period, the process of activation that was initiated with CO₂ apparently stops and waits for the light to initiate the second (light requiring) process. Since photo-activation is here suggested to occur at dawn, the second 12-hour period might be equivalent to Bünning's photophase. Now the hatching response to light exposure in the interval from hours 24 to 34 is the exciting thing since it strongly suggests an endogenous rhythm of recycling of the embryos to a state comparable to when the CO₂

tension was first elevated. Again CO_2 seems to activate a dark reaction and the embryo stores the light signal for dawn use. It is to be noted that the overall interval from light exposure to hatching is now shortened by approximately eight hours, almost as if in the first 24 hours some development had taken place in the presence of CO_2 but in the absence of light.

One essential demand of this interpretation is that CO_2 initiates a dark reaction and that initiation is independent of the time at which the embryos are introduced to the CO_2 elevation. To test this the same type of experiment as the preceding

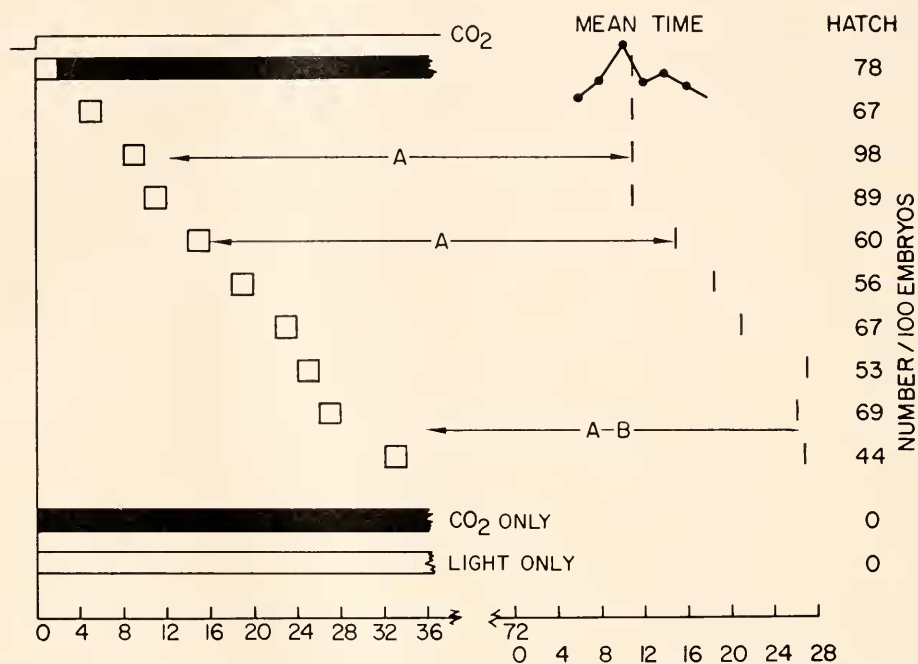


FIGURE 5. Mean hatching time of embryos exposed to a two-hour pulse of light at some time in a 34 hour interval following CO_2 elevation of the medium. Previously, embryos had spent three weeks in a N_2 environment following transfer from the "stream." "A" is the (variable) interval from onset of light to mean hatching time. "B" is the (fixed?) interval by which total development time (A) has been shortened in the second cycle following CO_2 elevation.

was repeated with four sets of embryos, with successive sets receiving the CO_2 elevation at hour 0, 6, 12 and 24. All sets were removed from the same container, which came from a stagnant medium, since the source in the flowing stream had been exhausted.

With one important exception the embryos responded as predicted. Following gasing the embryos began development which advanced them a maximum of 13 hours toward hatching and there appeared to be no qualitative difference in any of the four groups (Fig. 6). Unlike the results of the preceding experiment, the embryos continued to develop for the first 20 hours following gasing instead of for the first 12 hours. Also unlike the preceding experiment, all but the group

started at hour 12 began a reversal at hour 20 following CO₂ elevation. At hour 32 the groups started at hours 0 and 6 had reversed development to the point where hatching required the same duration as though light and the CO₂ elevation had been given simultaneously. Following hour 32 the two groups again began development toward hatching. Only the group started at hour 12 behaved similarly to the embryos in the preceding experiment in that development executed during the first 20 hours was retained throughout the subsequent 16 hours. With significant quantitative exceptions the experiment is believed to support the hypothesis that CO₂ triggers one of two essential processes necessary for termination of the diapause.

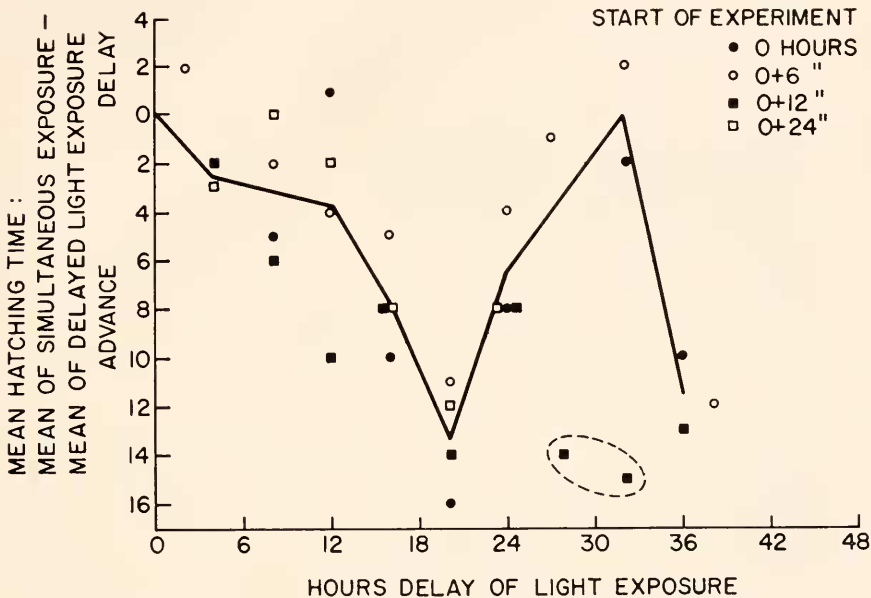


FIGURE 6. Mean hatching time of embryos given a two-hour light pulse simultaneous with CO₂ enrichment or at some time within 34 hours following enrichment. Four sets of embryos were treated with CO₂ at hour 0, or at 6, 12, or 24 hours later. Hatching time is expressed in hours from light exposure but relative to the hours required when both light and CO₂ treatments were given simultaneously. Embryos previously incubated for one year in a stagnant environment in constant dark.

The hypothesis also implies that the signal from the light pulse may be "stored" for initiation of the light requiring reaction following completion of the CO₂-initiated reaction. Experimental evidence is supportive. When CO₂ is withdrawn at hour 10 following the elevation, activation is not significantly greater than zero despite a light pulse given with the CO₂ elevation (Fig. 7). However, when withdrawal is followed immediately by a second light pulse (hour 10 to 12), activation is achieved and the embryos hatch. If four hours are allowed to elapse between withdrawal of CO₂ and exposure (hours 14 to 16) to light, no embryos are activated indicating that a CO₂ controlled process is reversible. These results strongly support the demand for induction of two temporally separate processes,

one of which is satisfied by CO_2 and the second of which requires light. They also show the order in which the two inducible processes must be satisfied when CO_2 is employed to break the photo-refractory phase of diapause.

Breaking diapause with modification of the gaseous environment

When the embryos are incubated in a "stream," enrichment of CO_2 concentration becomes a necessity for photo-sensitivity as shown above. CO_2 treatment was not sufficient by itself, however. Although the details are still being investigated,

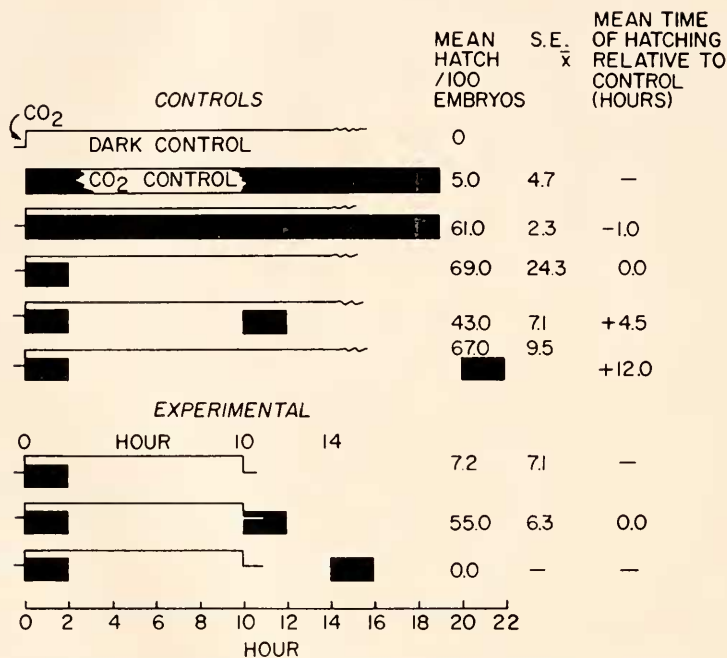


FIGURE 7. "Storage" of the light stimulus by CO_2 as shown by the need for a second light exposure when CO_2 is withdrawn following ten hours after CO_2 elevation and the initial light exposure.

the embryos require some preliminary exposure to a low O_2 environment. Following six months incubation in the "stream" a collection of embryos was divided into two groups. One group was subjected to a 100% N_2 environment for two weeks, the second to a 50% CO_2 , 20% O_2 environment for the same interval. Both groups of embryos were then exposed to light with or without CO_2 elevation (5% CO_2 in air). The groups treated with N_2 gave an 87.0 per cent hatch when exposed to both light and elevated CO_2 , the CO_2 control, 0.0 per cent. The groups treated with 50% CO_2 and normal O_2 gave no hatching in light and 5 per cent CO_2 , although there was a 6.0 per cent hatch in the CO_2 controls. The low O_2 (N_2) environment may be considered a prerequisite to the effectiveness of CO_2 elevation in the presence of light.

The low O₂ and high CO₂ environment, which occurs normally with aerobic metabolism in a closed system may therefore act independently in breaking the photo-refractory phase of diapause in *Daphnia*. Presumably this is what happens in a stagnant medium and ultimately results in the disappearance of one of the two inducible phases within the daily cycle of the embryo. Ballard and Grant Lipp (1969) have shown that a low O₂ atmosphere achieves partially the effect of an elevated CO₂ atmosphere in breaking the dormancy of clover seeds. A preliminary exposure to low O₂ was not necessary for CO₂ effectiveness in their material, however.

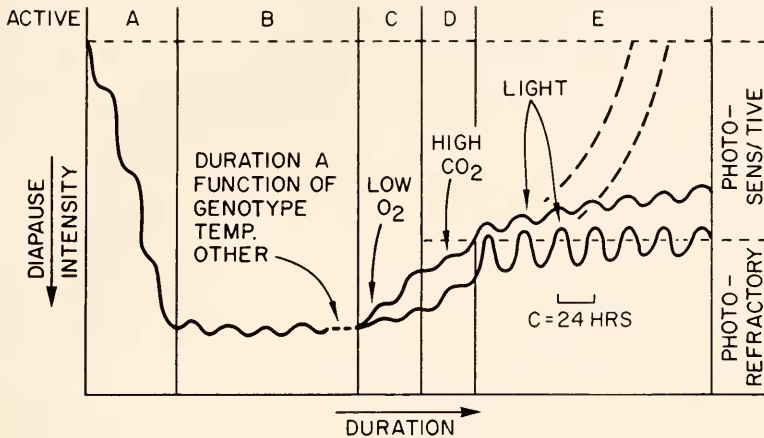


FIGURE 8. Schematic model of diapause interval in embryos of *Daphnia pulex*. Each of four states (A-D) are identified by the changing responsiveness of the embryos to specific stimuli in the environment, including temperature, O₂ and CO₂ tensions, and light. In a fifth state (E) the embryo is photosensitive. The light requirement for terminating the diapause may differ depending on the manner of entry to the photo-sensitive phase of diapause. (See text for details.)

A further complexity is that the *Daphnia* embryos are not immediately sensitive to the low O₂ stimulus. Some interval of incubation in the stream is a necessary preliminary condition for response to low O₂ after only two weeks. Four weeks of exposure was inadequate when the embryos were removed from the culture vessel and placed directly in low O₂. Preliminary experiments suggest that the embryos become primed for response to the low O₂ environment if the flow of medium in the stream is interrupted periodically.

Photo-refractoriness in the diapaused embryo may represent a variety of internal states, if responsiveness to the variety of stimuli employed in this and other studies may be used as criteria. A descriptive model (Fig. 8) of diapause suggests a minimum of five states, the first four (A-D) of which are photo-refractory. In the initial (A) state the diapause intensifies or deepens. During this state progressively fewer embryos are rendered photo-sensitive following such treatments as osmotic shock (Stross and Hill, 1968), decapsulation (Davison, 1969) etc. The following interval (B) is one of intense diapause, the duration of which

may be determined by temperature, genotype of the embryos, and, in the case of the strain used in this study, some unknown set of conditions.

A third state (C) is marked by the responsiveness of the embryo to low O_2 tensions, the effect of which is to induce a state (D) of sensitivity to a stimulus such as elevated CO_2 . The photo-refractory phase of diapause may be considered at an end when the "D" state has been satisfied. Since the light sensitizing action of CO_2 is reversible, the embryo may move reversibly from state C to state E, the final state, with the addition or withdrawal of CO_2 .

In the final or E state the embryo is fully photosensitive and exposure of the embryo to light at the appropriate phase of an endogenous cycle will trigger activation. The final state may be forced with manipulation of the gaseous environment, and it is suggested that only some strains of *D. pulex* are amenable, or it may result from incubation at the appropriate temperature.

Alternative states of photo-sensitivity in the "E" phase of diapause development are described in Figure 8. In one state (bottom line) the embryo is photo-inducible for only a part of a daily cycle which may include two (*e.g.*, Fig. 2) or one (*e.g.*, Fig. 3) phase of induction. In the alternative state (top line) the embryo may be continually sensitive to light during its "E" phase of diapause. Although sensitivity may be continuous, the rhythmic pattern of hatching (Figs. 5 and 6) would suggest that photo-induction is also rhythmic, that is, the product of a photo-reaction is apparently "stored" until needed in an internally ordered activation process.

DISCUSSION

The diapaused embryo of *Daphnia* is potentially under the control of photo-period, as may be the entire life cycle. The requirement for light is readily demonstrable. However the requirement for one inductively long day may be obscured by the internal state of the embryo. In one state induction clearly requires one long day or two pulses of light that simulate one long day. The embryo may therefore have two, not one, photo-inducible phases in each endogenous daily cycle. Scant evidence suggests that either the dawn or sunset stimulus may be received first.

In a second state only one light pulse of two hours is sufficient for inducing a return to active embryonic development. In this state the embryo is still manifesting time measurement since it is activated by the light pulse only at a certain time(s) or phase of a 24-hour thermocycle. The loss of one of two photo-inducible phases marks a second internal state and follows a first. Conceivably some arthropods have normally only one photo-inducible phase and its "loss" permits diapause termination in constant dark. There are many such examples of termination in constant dark and they include another strain of *Daphnia pulex* (Stross and Hill, 1968) as well as other arthropods, aquatic (Paris and Jenner, 1959) and terrestrial (Williams and Adkisson, 1964). That two photo-inducible phases may exist in arthropods other than *Daphnia* is demonstrated by the response of the aphid *Megoura* to photoperiod (Lees, 1966).

Other recent studies infer photo-induction of diapause termination at more than a single phase of a daily cycle. Hammer (1969), employing a regimen of

one short day followed by a cycle of complete darkness, shows that night interruptions in the first night period are more effective in creating a long-day than are light interruptions at the appropriate time in the second cycle. A light-influenced preparative process, the product of which decays following the end of the main light period, was inferred. Saunders (1970) using the same approach with a different insect described an opposite result in which interruptions during the second (and third) cycle were apparently more effective. Both studies suggest that two light controlled reactions may be involved in each inductive cycle.

The reaction to light at supposedly two phases, one at sunset and the other at dawn, is consistent with the response to night interruptions when given in 24-hour cycles. When the main light period is suitably short (6 to 10 hours) there are normally two intervals in the night when a light interruption is inductive. Exposure to light early in the night may be inductive when it forms the sunset of a long day. Exposures in the late night are effective when they form the dawn of a long day (Pittendrigh and Minis, 1964; Pittendrigh, 1966). Light exposure in the late-night is usually the more effective inducer of a long daylength (Adkisson, 1964, 1966; Saunders, 1968, 1970) presumably because the main light period now forms the sunset which may be interpreted as the phase of photo-inducibility. However, Pittendrigh (1966) strongly suggests the photo-inducible phase may be the late-night or dawn period, a view supported by the interpretation of the results in this paper. In that event the main light period would be involved with a second inductive phase which could be preparative for a second light reaction which may take place at dawn.

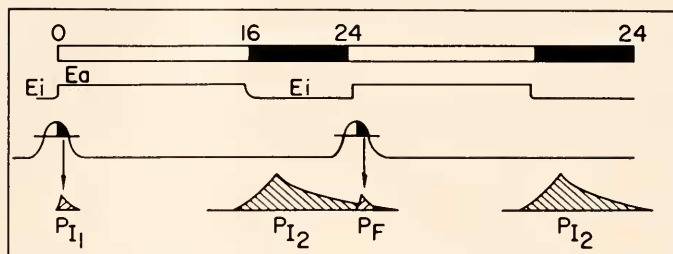
The existence of a second inducible phase that occurs near (sunset) the end of the light period and which provides an essential product for a photo-induction that occurs at dawn may be supported by temperature manipulation. Experiments that combine a period of chilling with night interruptions have shown that chilling during the day may interfere with the preparation while those given at night seem to preserve the product of the preparative process. At least such an interpretation may be deduced from the results as described by Saunders (1968) and Saunders and Sutton (1969).

The deliberate elevation of CO₂ tension, which may be necessary to break photo-refractoriness, introduces an additional complexity. The embryo now responds to a light stimulus as though it were continuously photo-inducible. However, the pattern of hatching suggests that the embryo may be photo-inducible only at certain phases of some internal cycle. To explain the continued sensitivity to light, it is suggested that some intermediate product is stored for a phase-specific process of activation. Storage apparently requires the presence of the elevated tension of CO₂.

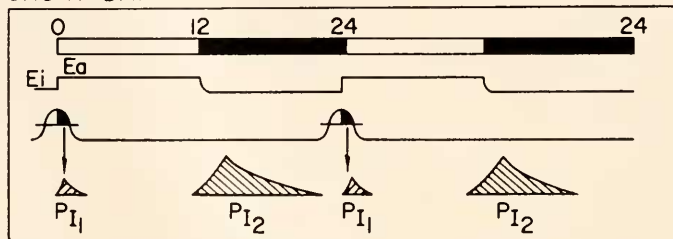
Three potential functions of CO₂ in terminating the diapause of *Daphnia* are described in an extension of the so-called coincidence model (Pittendrigh and Minis, 1964) (Fig. 9). One function is to break the photo-refractory state of the embryo. The second is to store a light signal. The third function is to preserve for all, or a part, of each daily cycle a product of some dark reaction until the embryo is exposed to light. The three (x, y, z) functions of CO₂ are deduced from the light requirements of the embryo in state one when there may be two photo-inductive phases each cycle.

The coincidence model describes photo-induction as a light activated enzyme converting a substrate when the concentration of the substrate achieves threshold concentration and that occurs once each circadian cycle. An extension (Fig. 9) describes two such substrate conversions, the products from each combining to form a final product. One intermediate product (P_{I1}) reacts with a second inter-

(A) LONG DAY



SHORT DAY



(B) CO_2 - INDUCED TERMINATION

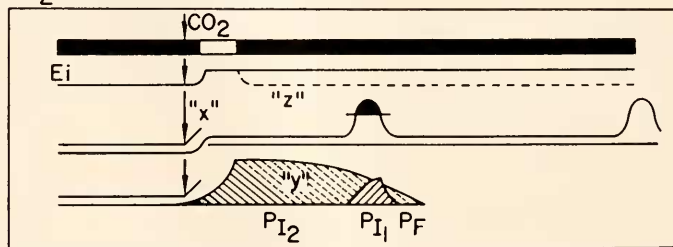


FIGURE 9. A model of photoperiodism describing the potential functions of CO_2 . The basic model is an extension of the coincidence model to include two "photo-inducible" phases in each daily cycle. Three functions of CO_2 are postulated to explain experimental observations. They are the breaking of photo-refractoriness ("x"), substitution of one light stimulus ("y"), and "storage" of a light signal until the embryo becomes photo-inducible ("z"). See text for details.

mediate (P_{I2}) to form the final product (P_F), provided the concentration of P_{I2} is sufficient, as would be the case if the night were short. Viewed in these terms, the potential functions of CO_2 are easily described.

The first function of CO_2 "unlocks" the machinery for making two intermediate products, shown in Figure 9B as the "x" function. This effect is analogous to excystment in parasites (Sommerville, 1964, 1966). The second function, "y,"

is to preserve the product of the dark reaction which provides the second substrate (P₁₂). This effect of CO₂ could be analagous to the preventative effect of CO₂ in short-day (long-night) induction (Barnett and Lilly, 1950, 1955) and possibly to the second function of stimulating molting in certain parasites. The third function, "z," is the presumed storage of the light signal until an essential substrate reaches critical concentration.

Whatever the functions of CO₂, it seems clear that termination of diapause in the *Daphnia* embryo may be controlled by daylength. Termination of the entire population in nature may therefore be synchronous. Synchrony is more likely to occur when the gaseous environment of the embryos is appropriately close to equilibrium with the atmosphere.

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SUMMARY

The diapaused embryo of the cladoceran, *Daphnia pulex*, may require light for terminating the diapause, and a single photocycle may be adequate. In *D. pulex* the light-refractory phase of diapause may be broken or completed in a variety of environments. In the Supply House strain employed in these studies, the usual low temperature treatment is unnecessary when the embryos are placed in constant darkness in sealed containers. Alternatively, the refractory state in constant darkness may be broken with low O₂ and high CO₂ tensions. Both modifications were shown to be necessary and in that sequence.

A single long-day light signal may terminate the diapause when the embryo passes from the photo-refractory to the photo-sensitive phase. Three kinds of light responses were observed, however. Each relates to the treatment given the embryos prior to light exposure.

In one so called internal state the embryo requires one long day or two pulses of light. Scant evidence suggests that the first pulse may be interpreted as either dawn or sunset. In this state there are obviously two photo-inductive phases in each inductive cycle. The embryo may change, however, by "losing" one of the photo-inductive phases. In this condition a single two-hour pulse of light, if given at the appropriate time of a thermocycle, may terminate the diapause.

A third internal state is introduced when CO₂ is employed to break the refractory phase of diapause. The diapause is terminated by a single two-hour pulse of light, and the embryo is continuously sensitive to the light stimulus. However, the pattern of hatching indicates that activation may occur at restricted phases within the induction cycle.

Several roles of CO₂ may be deduced from the experimental results. An elevated CO₂ tension breaks photo-refractoriness. It also induces a "dark"

reaction which may need to be completed before the light may stimulate termination of the diapause. Withdrawal of CO_2 after 10 hours prevents activation unless a second light pulse is given immediately thereafter. The apparent deferral of light stimulation suggests a third function of CO_2 , that of "storing" the light signal until the embryo becomes photo-inductive.

The potential roles of CO_2 are described in a model of photoperiodism that requires photo-induction at two phases in each daily cycle.

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